

Vorticella and *Colacium* as epibionts of copepods in Pasig river, Philippines

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Abstract

The chitinous exoskeleton of copepods serves as a scaffold for microepibionts such as protozoa. Although copepods are highly cosmopolitan, their associated epibionts in the tropics are poorly known. This study presents the first account in the Philippines of the ciliate *Vorticella* on calanoid copepod *Arctodiaptomus dorsalis* and both *Vorticella* and the euglenoid *Colacium* on the cyclopoid copepods *Thermocyclops crassus* and *Mesocyclops microlasius* in the Pasig River. Infestation, however, was low at 0.83% (108 out of 13,039) observed copepods from four collection sites in January-May and July-December 2018. Interestingly, *Vorticella* and *Colacium* were frequently observed in cyclopoid than on calanoid copepods, predominantly on the urosome, thorax, cephalosome of copepodites and adult stages of copepods. *Colacium* was also found attached on the antennules, swimming legs, caudal rami and setae. Prevalence of epibionts was site-related; *Vorticella* being more abundant in waters near Manila Bay (Site 1) where high salinity, conductivity and total dissolved solids were recorded while *Colacium* was found more near Laguna de Bay (Site 4). Both were abundantly present in September and absent in May, due to increased inflow of seawater from Manila Bay. Nevertheless, overall low infestation resulted in neither significant spatial nor temporal variation of epibiosis in Pasig River, probably due to heavy loadings of wastewater from nearby tributaries, noticeable algal blooms, detachment of epibionts by predations and changes in water chemistry.

Keywords: protozoa, zooplankton, epibiosis

Introduction

Protozoa are unicellular eukaryotic microorganisms found on or within other organisms, in decaying matter, soil and the aquatic environment (Kudo, 1939; Finlay & Esteban, 1998) by predated on bacteria and fungi (Hausmann et al., 2003). They are observed readily in microscopy as epibionts on planktonic microcrustaceans, such as copepods. Global records, generally from temperate and subtropical countries, indicate that copepods' chitinous exoskeleton is a favored niche of several protozoa like peritrich ciliates *Vorticella*, *Epistylis*, *Zoothamnium*, *Trichodina*, *Scyphidia* and *Rhabdostyla*,

suctorians *Pelagacineta*, *Euphelota*, *Paracineta*, *Ellobiopsis*, and *Tokophyra*, apostome *Vampyrophyra* and flagellate *Colacium* epibionts (Green & Sheil, 2000; Burris & Dam, 2014; Gregori et al., 2016; Walkusz & Rolbiecki, 2007; Regali-Selegim & Godinho, 2004; Clamp et al., 2016; Chiavelli et al., 1993; Ohtsuka et al., 2004). Other host substrates include cladocerans, isopods, decapods and other macrocrustaceans (Fernandez-Leborans & Tato-Porto, 2000). The density and preferred attachment sites of epibionts affect the mobility, reproduction and survival of the host (Regali-Selegim & Godinho, 2004). The paper by Taniguichi et al. (1999), to our knowledge, is the lone Philippine occurrence record of epibionts on copepods (haracticoida, calanoids, cyclopoids), ostracods and cladocerans. It documents with scanning electron micrographs (SEM) the predominance of unidentified rod-shaped bacteria, microalgae and protozoa on these microcrustaceans found in overlaying irrigation water in rice paddies.

Our study explores in-depth and for the first time the spatial and temporal epibiosis of two protozoa on copepods in

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Pasig River, a highly eutrophic and complex water system with a seasonal shifting bidirectional flow and mixing of freshwater and seawater coming from opposite ends. Pasig River (Luzon Is., Philippines) is a 27-km water system with a serpentine route flowing westward from Laguna de Bay, through Metro Manila to Manila Bay, the outlet facing the West Philippine Sea (Fig. 1). Laguna de Bay itself is heavily eutrophic, and the quality of its water downstream in Pasig River to Manila Bay is impacted by added inputs from four major and forty-seven minor tributaries and by different domestic and industrial pollutants from urban Metro Manila (Cruz, 1997). In sum, water in Pasig River is environmentally complex physically, chemically and biologically (Petts, 1986). Water quality is a measure of pollution level, which affects the diversity and abundance of zooplankton (Dorak, 2013). Our study focuses on the frequency and distribution of protozoa *Vorticella* and *Colacium* as epibionts on the body of host copepods, and their occurrence in relation to the physico-chemical factors in Pasig River in 2018.

Materials and Methods

Study sites

Four sampling sites stretched eastward along the Pasig River were selected for the study (Fig. 1). Site 1 (40°35'42.57"N; 120°57'48.16"E) was situated at Tacoma Port Area, Manila near the mouth of the river where vessels load and discharge cargos of commerce. Site 2 (14°35'18.83"N; 121°00'46.74"E) was at Santa Ana Port, Manila near the junction of San Juan River. It was surrounded by residential and industrial complexes, as is the case at Site 3 (14°33'43.9"N; 121°03'45.01"E) in Pineda, Pasig City near Marikina River. Site 4 (14°32'03.92"N; 121°06'16.74"E) was the closest to Laguna de Bay and at the boundary of Rizal Province and Pasig City.

Field sampling

Samples were collected monthly from January to May 2018 to determine the spatial variations of the physico-chemical features across the river and the occurrence of epibionts on copepods. Sites with significant variation in terms of physico-chemistry data and frequency of copepods with epibionts were selected for further analysis in July to December 2018. No sampling was done in June due to inclement weather.

Samples for plankton were obtained with a use of 90-µm mesh plankton net. Collection was done in replicates. The collected samples were placed in 500 ml containers and transported to the laboratory for further processing. Water quality parameters such as pH, salinity, TDS, temperature, dissolved oxygen and conductivity were measured *in-situ* using ExStik DO600, ExStik EC500 and Hach HQ40d portable water

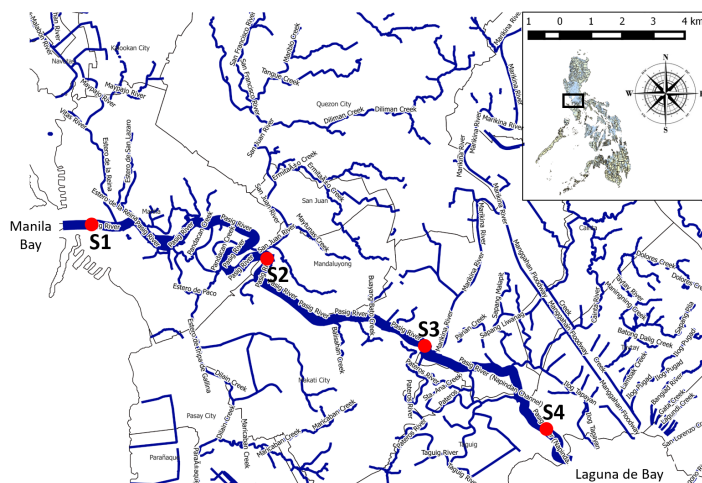


Figure 1. Location of the different sampling sites: S1-S4 represent sites along Pasig River

quality meters. DO was not recorded in Sites 1 and 3 due to authorities' restriction and site risk.

Copepod identification and enumeration

Distinctive morphological structures such as antennule length and segmentation were considered to differentiate copepods into orders Calanoida and Cyclopoida (Fernando, 2002) and thereafter, to species following Dussart & Defaye (2011).

Copepods with and without protozoan as epibionts were sorted out in separate containers, and level of infestation was computed.

Enumeration, examination and identification of copepod epibionts

Infested live copepods were segregated and examined using Optika SZM-1 dissecting microscope and Olympus CX21 compound microscope for the numerical counts of epibionts and their attachment on the host body parts-antennule, cephalosome, segmented thorax and urosome, swimming legs, caudal rami and setae. Noted also were other morphological structures of epibionts: body shape and length, cilia and stalk. Identification of epibionts was based on the works of Foissner & Berger (1996), Kudo, (1939) and Prescott (1951).

Imaging

Phase contrast micrographs were taken using B-350 Optika Microscope (Italy). Some infested specimens were fixed with 2% glutaraldehyde and dehydrated through graded series of ethanol and air dried prior to observation under Hitachi Table-Top scanning electron microscope (Taniguchi et al., 1999).

Data analysis

The significance of the percentage distribution of epibionts across the developmental stages of copepods was analyzed using one sample t-test and the frequency distribution of epibionts by Chi-square Goodness of Fit. Physico-chemistry data from January to May 2018 were analyzed using two-way ANOVA followed by Tukey HSD post hoc test. One-way ANOVA and independent sample t-test were used to analyze data recorded at Sites 1 and 4 from July to December 2018 using IBM SPSS Statistics version 20. Principal Component Analysis was used to evaluate the variation of the average values of the measured physico-chemical parameters from Pasig River over an 11-month period of sampling at Sites 1 and 4 through PAST 3.20. Figure showing the percent distribution of infested and non-infested copepods was generated using Sigma plot.

Results

Copepods and their epibionts in the Pasig River

Three species of copepods, namely: *Arctodiaptomus dorsalis* (Marsh, 1907) [Calanoida] and *Thermocyclops crassus* (Fischer, 1853) and *Mesocyclops microlasius* Kiefer, 1981 [both Cyclopoida] were collected from the Pasig River. Of the 13,039 copepods examined, only 108 copepods were infested (1 calanoid and 107 cyclopoids). One individual of *A. dorsalis* was seen infested by *Vorticella* while *T. crassus* and *M. microlasius* were found to be infested with *Vorticella* and *Colacium*. Among the infested cyclopoids, 27 had *Vorticella*, 57 had *Colacium* and 23 with both epibionts.

Vorticella (phylum Ciliophora) and *Colacium* (phylum Euglenozoa) were the only protozoan epibionts found on the copepods. *Vorticella* was observed abundantly on the urosome, thorax and cephalothorax in decreasing order both in copepodite and adult stages. In contrast, it was not found on the swimming legs and antennules (Fig. 3). Further, *Vorticella* was not equally distributed on the body of the copepod across CI (χ^2 (2, n = 20) = 7.900, p = 0.019), CII (χ^2 (2, n = 20) = 7.900, p = 0.019), CIII (χ^2 (2, n = 29) = 8.345, p = 0.015), CIV (χ^2 (2, n = 27) = 21.440, p < 0.001), CV (χ^2 (2, n = 27) = 8.667, p = 0.013) and adult (χ^2 (2, n = 117) = 86.000, p < 0.001). For CI, *Vorticella* was found limited on the segmented thorax (n =5) (Fig. 2). Infestation by *Colacium* was also observed in the same copepod body harboring *Vorticella*, but more abundantly on the cephalosome of adults and on the thorax for copepodites. In addition, it also attached on the antennule, legs, caudal rami and setae of copepods (Fig. 4). In contrast with that of *Vorticella*, *Colacium* distribution on the copepod body was found to be insignificant in CI (χ^2 (4, n = 14) = 4.571, p = 0.334) and CV (χ^2 (3, n = 44) =



Figure 2. Percent distribution of *Vorticella* and *Colacium* across copepods' body segments in every copepodite and adult stages

6.727, p = 0.081) but significant in CII (χ^2 (4, n = 260) = 103.346, p < 0.001), CIII (χ^2 (5, n = 312) = 241.654, p < 0.001), CIV (χ^2 (5, n = 118) = 89.356, p < 0.001) and adult (χ^2 (5, n = 38) = 27.053, p < 0.001).

Epibionts were never seen in copepod nauplii. However, one sample t-test revealed that there was a significant difference on the percent distribution of epibionts across the copepodite and adult stages (t (5) = 4.11, p = 0.009, d = 1.67). *Vorticella* was mostly found on adults as compared to copepodite

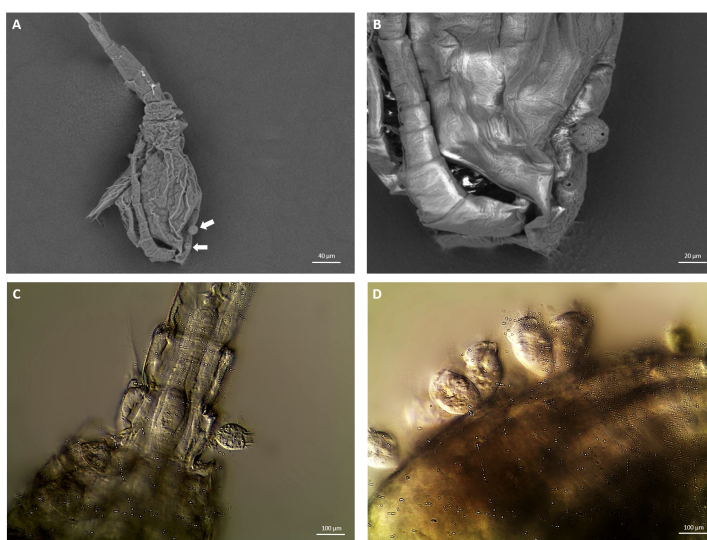


Figure 3. *Vorticella* (A) SEM of cyclopoid with *Vorticella* (B) magnification of *Vorticella* on cephalosome of cyclopoid (C) Phase contrast micrograph of *Vorticella* on urosome and on the (D) thorax segments

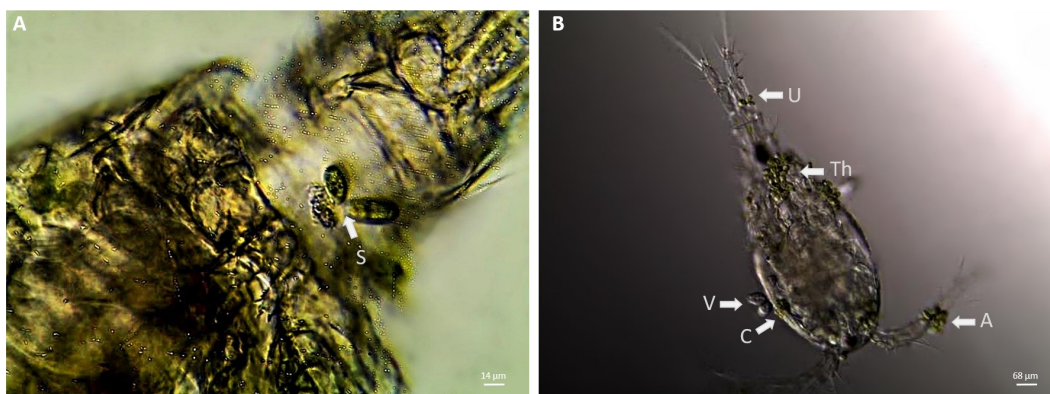


Figure 4. *Colacium* (A) Phase contrast micrograph of *Colacium* attached through its stalk on the urosome, (B) U-urosome, Th-thorax, C-cephalosome, A-antennule with V-*Vorticella*

copepods. In contrast, *Colacium* was mostly found on copepodite specifically on CII and III compared to adult (Fig. 2).

Imaging

Specimens of *Vorticella* in SEM micrographs had an inverted bell-shaped body (mean \pm standard error, SE), $95.87 \pm 7.00 \mu\text{m}$ for length and $60.02 \pm 3.53 \mu\text{m}$ for width. The peristome had a thick persitomial collar, circular central peristomial disc, transverse parallel pellicle striations (Fig. 5), prominent buccal ciliature in the peristomial region (Fig. 3) and erect outer ciliary row. Other details of the buccal region were not shown in the SEM; however, the stalk was seen in its contracted state. Live *Vorticella* was readily recognized when it responds to stimuli by the sequential shortening and lengthening of its contractile stalk. Euglenoid *Colacium* had a green ellipsoid body, $14.92 \pm 0.63 \mu\text{m}$ long with rounded tip and $8.58 \pm 0.31 \mu\text{m}$ wide; within were numerous discoid chloroplasts. It occurred singly and had a short transparent stalk attached to but

with no apparent movement on the host. The flagellum was not seen using the SEM.

Variation in epibionts across space and time

The occurrence of epibionts on copepods showed no significant variation in terms of its spatial or temporal distribution due to its low prevalence. The highest prevalence of epibionts was 3% in September 2018 (Fig. 6) and the mean prevalence for the whole sampling was 0.83%.

There was a variation between the four sampling sites from January to May based on the physico-chemistry data: pH (F (12, 40) = 129.812, $p < 0.001$), salinity (F (12, 40) = 1340.136, $p < 0.001$), conductivity (F (12, 40) = 237.747, $p < 0.001$) and TDS (F (12, 40) = 502.117, $p < 0.001$) from January to May. Post hoc Tukey HSD multiple comparison test revealed that Site 1 varied significantly in all sites in terms of TDS, conductivity and salinity. The mean values of TDS ($1318.28 \pm 285.95 \text{ mg/L}$), conductivity ($1734.07 \pm 340.08 \mu\text{S/cm}$) and salinity ($998.93 \pm 201.29 \text{ ppm}$) were all highest in Site 1 as compared with the lower values in Site 4 for TDS ($331.95 \pm 5.51 \text{ mg/L}$) and conductivity ($470.05 \pm 8.59 \mu\text{S/cm}$) and salinity ($273.19 \pm 23.82 \text{ ppm}$). The mean pH values across the four sampling months increased from Sites 1 to 4 ranging from 7.22 ± 0.03 – 7.53 ± 0.07 . The highest mean temperature value of $30 \pm 0.59 ^\circ\text{C}$ was recorded in Site 1 and lowest in Site 4 with $27.19 \pm 0.76 ^\circ\text{C}$. DO concentration was higher in Site 4 as compared to Site 2 with mean values of $4.23 \pm 0.91 \text{ mg/L}$ and $1.71 \pm 0.63 \text{ mg/L}$, respectively (Fig. 7). Interestingly, copepods with epibionts were found in Site 1 and none in Site 4. This led to the selection of Sites 1 and 4 for further sampling in July to December 2018. No further study was conducted in Sites 2 and 3 since copepods with epibionts only occurred once in Site 3 and none in Site 2. Salinity (F (5, 30) = 3.512, $p = 0.13$), conductivity (F (5, 30) = 91.018, $p < 0.001$), TDS (F (5, 30) = 3.018, $p = 0.025$) and temperature (F (5, 30) = 23.176, $p < 0.001$) varied significantly

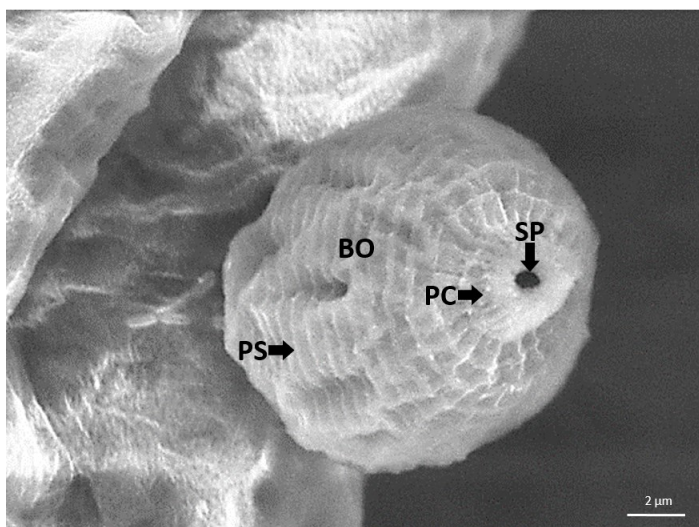


Figure 5. *Vorticella* showing captured parts under SEM

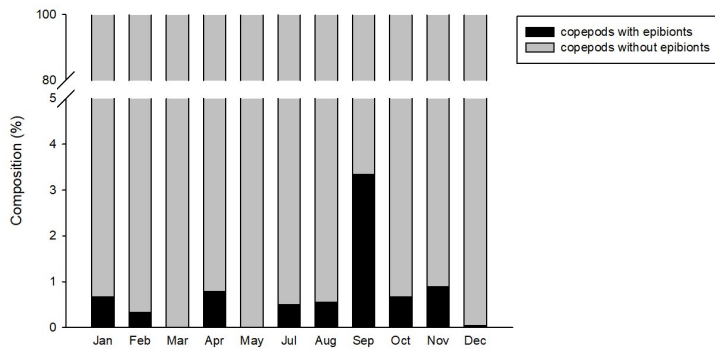


Figure 6. Percent composition of copepods with epibionts among the collected copepods in all selected sites in Pasig River.

from July to December. pH ($t(34) = -2.281$, $p=0.029$), salinity ($t(34) = 3.576$, $p=0.001$) and TDS ($t(34) = 3.588$, $p=0.001$) varied significantly between Sites 1 and 4.

PCA bi-plot revealed that average pH, salinity, TDS and conductivity were the parameters contributing to the variation among Sites 1 and 4 during the 11 sampling months (Fig. 8). PC1 (61.47 %) and PC2 (24.799 %), with eigenvalues >1 , accounted for 86.27% of the total variance. Salinity, conductivity and TDS had high loadings on PC1 while pH was high on PC2 (Table 1).

There were 54, 3 and 51 copepods with epibionts recorded in Sites 1, 3, and 4, respectively. Only *Vorticella* were observed in Sites 1 and 3 and during the months of January, February and April. There were no recorded copepods with epibionts during the March and May collections. It was noticeable that phytoplankton such as cf. *Melosira* spp. and *Microcystis* spp. heavily dominated the river during March to April. It was also observed that copepods with epibionts were present in January to February 2018 during the outflow of water from Laguna de Bay and absent in March and May during the inflow of water from Manila Bay. *Vorticella* was recorded once in Site 1 in July and none in Sites 1 and 4 in August. Instead, *Colacium* spp.

Table 1. Factor loadings based on the principal component analysis for various water physico-chemical parameters

Physico-chemical parameter	PC1	PC2
% variance explained	56.178	23.411
pH	-0.168	0.690
Sal (ppm)	0.562	0.132
Con ($\mu\text{S}/\text{cm}$)	0.558	0.148
TDS (mg/L)	0.560	0.144
Temp ($^{\circ}\text{C}$)	0.178	-0.681

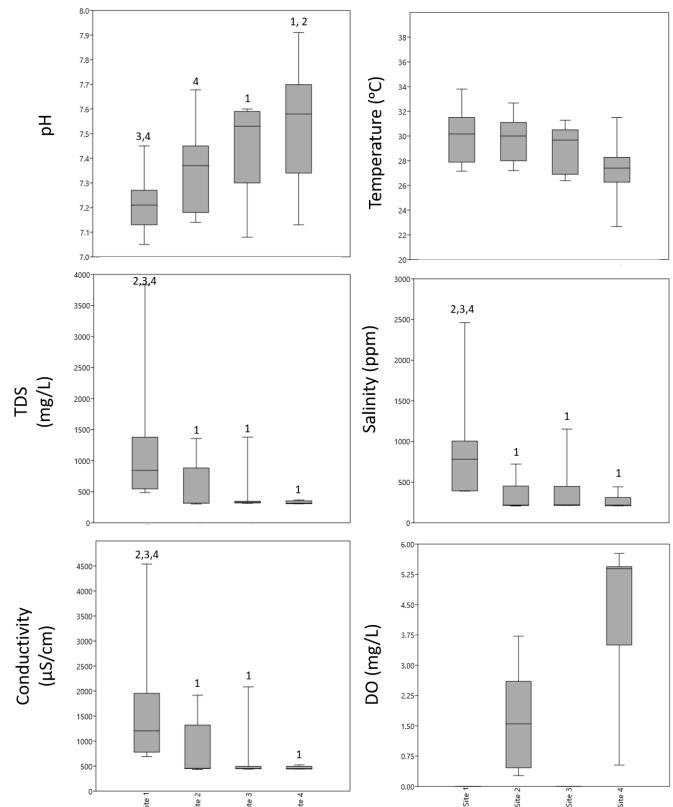


Figure 7. Box plot of physico-chemical parameters along the sampling sites from January to May 2018 *The numbers above bars indicate significant differences across the sites ($p>0.05$) based on Tukey HSD post hoc test

were frequently found as copepods' epibiont in July and the only epibiont in August and they occurred in both Sites 1 and 4. Both *Vorticella* and *Colacium* were observed abundantly in September and declined in October to December 2018.

Discussion

Occurrence of epibionts on copepods

Thermocyclops crassus, a species complex and widely distributed in Eurasia, Australia and the Americas, and *Mesocyclops microlasius*, endemic in the Philippines with records in Luzon Is. including Manila, Laguna and Rizal (Lopez et al., 2017) are herein recorded for the first time in Pasig River. In addition, *Arctodiaptomus dorsalis*, which is considered as an invasive species in Philippine lakes and was first recorded in Laguna de Bay (Papa et al., 2012), was also recorded in the river. The widespread distribution of these copepods may be the reason of their potential as substrate for epibiotic microorganisms.

The chitinous exoskeleton of copepods served as the

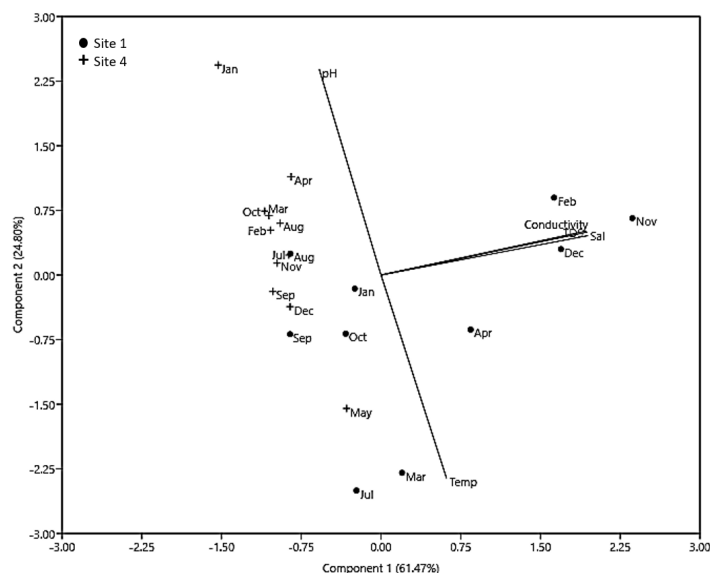


Figure 8. PCA bi-plot of the physico-chemical parameters of Sites 1 and 4 during the 11 month sampling in Pasig River

substrate for *Vorticella* and *Colacium*. Although both genera were observed as epibionts of cyclopoids, they interestingly showed similar points of attachment sites such as on the urosome, thorax and cephalosome and absence on some parts. *Vorticella* were found abundantly on the urosome since this segment lacks swimming legs that could interfere with their attachment. Also, food particles left by copepods may be directed towards the attached epibionts on the urosome and thorax regions. Attachment on the thorax and urosome may be favorable to both genera due to the lesser dragging force of water as compared when attached to the upper segment of the copepods. However, in the case of *Colacium*, the cephalosome was also a good site of attachment since it has greater surface area and could probably allow more epibionts to attach and easily establish a point of attachment considering that the host and the epibionts are motile. Other epibionts such as *Paracineta* sp. and *Epistylis niagarae* Kellicott, 1883 also had prosome and urosome as their points of attachment on *Metridia longa* (Lubbock, 1854), *Paraeuchaeta norvegica* (Boeck, 1872) and *Mesocyclops aspericornis* (Daday, 1906), respectively (Walkusz & Rolbiecki, 2007; Rajabunizal & Ramanibai, 2011). On the other hand, only *Colacium* were seen on the swimming legs, caudal rami and antennule of cyclopoids. This was also observed on *Tigriopus fulvus* (Fischer, 1860) but with another epibiont, *Scyphidia* (Pane et al., 2014). This was never the case of *Vorticella* in this study. This may be caused by the prominent elongated stalk that *Vorticella* possesses as compared to *Colacium* and *Scyphidia*. Its stalk also contracts and covers greater area. Due to the size and motility of *Vorticella*,

swimming and feeding process of copepods may be hindered and may not withstand its movement. This suggests that attachment of epibionts vary and may depend on the size and habits of both the host and the epibionts.

Attachment of protozoa to copepods is facultative and may be associated with the mortality and molting of the host (Walkusz & Rolbiecki, 2007). Both *Vorticella* and *Colacium* were found on copepodite and adult stages and never in naupliar stages which have a higher molting rate (Utz and Coat, 2005). *Vorticella* were mostly recognized on adult since molting occurred in copepodite stages. Contradicting to this case is *Colacium* wherein it was mostly observed in copepodite stages. This may be due to its capacity to attach until its substrate dies or dries up (Willey et al., 1990). Attachment of *Colacium* on adult copepods would further increase its size and result in an increase of its visibility to predators.

Epibionts and their environment

The occurrence of epibionts on copepods showed no correlation to the prevailing physico-chemical conditions of Pasig River due to their low prevalence. The 0.83% prevalence of epibionts on copepods computed in this study was even lower than the computed low prevalence value of 3.3% of *Epistylis* sp. and *Synedra* sp. on calanoids and cyclopoids (López et al., 1998). Low occurrence of epibionts may suggest that other biotic or abiotic substrate might support *Vorticella* and *Colacium*, although attachment on live moving substrates could be beneficial. Other factors such as bacterial population and other water quality parameters such as depth and nutrient loadings which were not included in this study might be some factors that could further explain their presence and absence in each site and sampling months. It was reported that species of *Vorticella* can withstand acidic water with pH less than 3.0 (Packroff & Woelfl, 2000), temperature ranging from 0-30 °C and dissolved oxygen ranging from 0-16 mg/L (Bick, 1972). *Colacium* was considered to be eurythermal and can thrive in an environment with pH value between 6 and 9 and dissolved oxygen levels between 3.6 and 10.4 mg/L (Ersanli & Gonulol, 2003). The wide range of values suggests the adaptability of the epibionts in Pasig River. However, the duration and fluctuation of their exposure to such conditions may be the contributing factor that can trigger changes in its life phase and cause detachment.

Due to changing and stressful aquatic conditions, adhesion to a body surface that serves as a living support to epibiotic microorganisms becomes necessary for survival (Roszak & Colwell, 1987). It was observed that *Vorticella* detached from copepods. *Vorticella* developed into a telotroch that swims and settles on a substrate by attaching its stalk and further develops

into its adult stage. It tends to develop into a telotroch stage again and searches for another substrate where it could attach by growing over its stalk if the environment is unfavorable (Verma & Jordan, 1995). In the case of *Colacium*, from its free-living flagellated cell, it develops a stalk and adheres to a substrate until the substrate become unstable. It re-extrudes its flagellum and finds another substrate to attach on (Rosowski & Kugrens, 1973). Both have vegetative stages that would require substrate for them to survive.

Because of their cystic, free-living stages and diminutive size, sampling and microscopy of protozoa hinder the study of their occurrence on copepods despite their abundance in the microbial community. Furthermore, observation of protozoa on live copepods was one of the challenges in this study. Nevertheless, this study presents the first account of the occurrence of *Vorticella* and *Colacium* as epibionts of copepods in Pasig River and in the Philippines. Both genera were found to attach frequently in the cephalosomal, thoracic and urosomal regions of copepods but only *Colacium* were found to attach on the antennule, swimming legs, caudal rami and setae. Their occurrence may not be explained by the environmental condition of the Pasig River but the effects of their exposure to the measured conditions may be a significant study. Moreover, this study contributes to the limited investigation of epibionts on copepods in the country and in the tropical regions.

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